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Repeated evolution of asexuality involves convergent gene expression changes

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Abstract

Asexual reproduction has evolved repeatedly from sexual ancestors across a wide range of taxa. While the costs and benefits associated with asexuality have received considerable attention, the molecular changes underpinning the evolution of asexual reproduction remain relatively unexplored. In particular, it is completely unknown whether the repeated evolution of asexual phenotypes involves similar molecular changes, as previous studies have focused on changes occurring in single lineages. Here we investigate the extent of convergent gene expression changes across five independent transitions to asexuality in stick insects. We compared gene expression of asexual females to females of close sexual relatives in whole-bodies, reproductive tracts, and legs. We identified a striking amount of convergent gene expression change (up to 8% of genes), greatly exceeding that expected by chance. Convergent changes were also tissue-specific, and most likely driven by selection for functional changes. Genes showing convergent changes in the reproductive tract were associated with meiotic spindle formation and centrosome organization. These genes are particularly interesting as they can influence the production of unreduced eggs, a key barrier to asexual reproduction. Changes in legs and whole-bodies were likely involved in female sexual trait decay, with enrichment in terms such as sperm-storage and pigmentation. By identifying changes occurring across multiple independent transitions to asexuality, our results provide a rare insight into the molecular basis of asexual phenotypes and suggest that the evolutionary path to asexuality is highly constrained, requiring repeated changes to the same key genes.

Keywords: convergent evolution, parthenogenesis, *Timema*

Sexual reproduction is extremely costly. Sex is less efficient than asexuality for transmitting genes to future generations (Maynard Smith 1971) and in order to outcross, an individual has to find a partner, forgo foraging, and risk contracting sexually transmitted diseases and predation while mating (Bell 1982; Lehtonen et al. 2012). Yet, the overwhelming number of sexual, as compared to asexual, animal and plant species (Avise 2008; van der Kooi et al. 2017) indicates that sexual reproduction is highly advantageous. Identifying potential advantages conferred by sex has motivated decades of research and a rich body of work on the evolution and maintenance of sexual and asexual reproduction has been produced (reviewed in Bell 1982; Lewis 1987; West et al. 1999; Otto 2009; Neiman et al. 2017). By contrast, little is known about the molecular underpinnings required to evolve asexuality from sexual ancestors (Neiman et al. 2014). Yet these molecular underpinnings have the potential to provide insights into the processes involved in the evolution of asexuality, and to help understand how sex is maintained. For example, sex is more easily maintained if asexuality evolves gradually in a sexual population than if it emerges suddenly via major effect mutations (Templeton 1982; Burt 2000; Schwander et al. 2010).

Some insight into the genetic basis of asexuality has been gained from studies of individual asexual lineages (Innes and Hebert 1988; Lynch et al. 2008; Eads et al. 2012; Jaquiéry et al. 2014), but a broad comparative framework for exploring common principles of the molecular basis of asexuality is lacking. For example, a major unresolved question is whether independent transitions to asexuality involve similar or different molecular changes. To address these shortcomings, we explored the molecular underpinnings of asexuality in stick insects of the genus *Timema*, a genus of wingless, herbivorous insects native to the West coast of North America and the mountains of the Desert Southwest. This group is uniquely suited for comparative studies of asexuality, as asexuality has evolved at least seven times independently (Schwander et al. 2011) (Fig. 1), allowing us to study convergence across replicate transitions from sexual to asexual reproduction. Furthermore, close sexual relatives are at hand for each asexual lineage for comparison. All asexual *Timema* species reproduce via obligate parthenogenesis (Schwander and Crespi 2009), meaning that they evolved the ability to produce unreduced eggs which develop without fertilization by sperm. Additional phenotypic changes evolved convergently as adaptations to asexuality, including a reduced sperm storage organ, and reduced sexual pheromone production (Schwander, Crespi, et al. 2013). Thus, asexual *Timema* females are less attractive to sexual males (Schwander, Crespi, et al. 2013), which use both airborne and contact signals to identify suitable mates (Nosil et al. 2007; Arbuthnott and Crespi 2009; Schwander, Arbuthnott, et al. 2013), and even when copulations between sexual asexual females and males from sister-species are forced under laboratory conditions, eggs are not fertilized (Schwander, Crespi, et al. 2013).

To capture molecular changes associated with the evolution of asexuality we performed whole-body and tissue-specific transcriptome sequencing (RNA-seq) on females from five sexual and five asexual *Timema* species (Fig. 1). We chose two different tissues, the reproductive tract and legs, to identify the molecular mechanisms underlying the production of asexual offspring (reproductive tract), and adaptations to a celibate life (e.g. reduction of various different sexual traits in the reproductive tract and legs). Note that the reproductive tract and leg samples actually represent a collection of tissues, but we refer to them as tissues throughout for brevity. Whole-

86 body samples were included as they allow us to identify important changes that may be missing
87 in the tissue-specific transcriptomes. Using this approach, we identified convergent expression
88 changes which were likely driven by selection. We also observed changes specific to each sexual-
89 asexual species-pair which typically showed concerted changes across tissues, consistent with
90 being a product of drift (Blekhman et al. 2008; Liang et al. 2018). Finally, to complement our
91 expression analyses, we examined patterns of molecular evolution in genes showing convergent
92 expression changes following a transition to asexuality.
93

Results

Transcriptomes and orthology

Reference transcriptome assemblies for each species were generated previously (Bast et al. 2018). Bast et al. (2018) also identified 3010 one-to-one orthologs, which were used as our transcriptome reference. For each tissue, orthologs with low expression (counts per million less than 0.5 in two or more libraries per species) were filtered prior to expression analyses. Thus, the final number of orthologs kept for analyses of whole-body, reproductive tract, and leg samples was 2984, 2753, and 2740, respectively.

Convergent gene expression changes

We identified convergent gene expression changes between sexual and asexual species by modelling gene expression as a function of species-pair (see Fig. 1), reproductive mode (sexual or asexual), and their interaction in edgeR (Robinson et al. 2010). In such a model, convergence is indicated by an overall effect of reproductive mode (FDR < 0.05), but no interaction (FDR > 0.05) (Supplemental Table 1). Approximately four times as many genes changed convergently in the reproductive tract (7%; 203/2754) and legs (8%; 206/2737) as compared to the whole-body (2%; 57/2985), perhaps reflecting the relative difficulty in identifying expression changes in complex tissue assemblies such as whole-bodies (Johnson et al. 2013). The amount of convergence we observe is considerable and approximately double what we would expect by chance, for all tissues (whole-body: $p = 0.0128$, reproductive tract: $p < 0.0001$, legs: $p < 0.0001$, Supplemental Fig. 1). The amount of change between sexual and asexual females was relatively small for convergent genes, with a mean fold change of approximately 1.4 (absolute $\log_2$ expression change for whole-body = 0.55, reproductive tract = 0.68, and legs = 0.46) (Fig. 2).

As expected for selection-driven gene expression changes (Blekhman et al. 2008; Liang et al. 2018), convergent changes between sexual and asexual species were highly tissue-specific. Only 22 of the convergent genes in the reproductive tract (203) and legs (206) overlapped between the two tissues, a value not significantly greater than expected by chance (Table 1). There was also little overlap between convergent genes in the two tissues and whole-bodies (Table 1, Supplemental Fig. 2). This supports the interpretation that the convergent changes in expression are driven by selection rather than by drift, as drift is more likely cause similar changes across multiple tissues. This interpretation of selection also predicts that convergent genes will be involved in divergent functions in each tissue which, as we show below, is indeed the case.

To directly test whether the convergent changes are driven by selection, we modeled convergent change as an Ornstein-Uhlenbeck (OU) process. Selection-driven expression changes are identified in this framework by comparing the likelihood of two models: a drift-model where expression changes are modelled as simple Brownian motion, and an adaptive-optima model where expression changes are modelled as the result of both Brownian motion and selection toward specified adaptive-optima (see Butler and King (2004) and Cressler et al. (2015)). In this case the second model specified that asexual species had a different adaptive-optimum than

138 sexual species. Using this approach, we found that the adaptive-optima model had a significantly
139 better fit than the drift model for 70% to 80% of convergently expressed genes, depending on
140 tissue (Supplemental Table 2, FDR < 0.05). This strengthens our interpretation that the majority
141 of the convergent changes between sexual and asexual species are driven by selection.
142
143 ***Functional processes of convergently expressed genes***
144
145 To detect convergence at the process level, we performed gene set enrichment analyses (GSEA)
146 for each tissue separately. Briefly, we scored Gene Ontology (GO) terms according to the rank of
147 convergent expression change of genes annotated to the terms; GO terms were then called
148 significant if they had a better average rank than expected by chance (see Methods). For each
149 tissue, more than 100 GO terms are enriched (FDR < 0.05), providing strong support for
150 convergence of biological processes between asexual species (Supplementary Tables 3-5). This
151 signal is not dependent on any threshold at the gene level, and thus provides information on
152 convergence at the process level due to small but consistent contributions from many genes.
153 Consistent with the gene expression results, enriched GO terms were generally tissue specific;
154 we found no significant overlap between GO-terms enriched in the legs and reproductive tract (11
155 shared terms, FDR = 0.123), between the legs and whole-body (4 shared terms, FDR = 0.799),
156 or between whole-body and reproductive tract samples (10 shared terms, FDR = 0.064).
157
158 To reduce the number of enriched GO terms to examine we semantically clustered enriched GO
159 terms using ReviGO (Supek et al. 2011) (Supplemental Tables 6-8). The annotations of
160 convergent changes in the reproductive tract reflect the convergent evolution of parthenogenesis
161 in asexual *Timema*, as they were linked to meiosis (meiotic spindle organization, meiosis II,
162 centrosome duplication, meiosis I cytokinesis, meiosis II cytokinesis), and reproduction (growth
163 of a germarium-derived egg chamber, sperm individualization, gamete generation). However,
164 convergent changes were also linked to neuron development (neurogenesis, neuron
165 development, neuron recognition), as well as several GO terms involved in development and
166 metabolic processes for which the link to asexuality is less clear. In legs we identified GO terms
167 involved in immune defence (response to fungus, regulation of production of molecular mediator
168 of immune response, regulation of antimicrobial peptide production, regulation of humoral immune
169 response), which may be because asexual females are no longer susceptible to the costs
170 associated with diseases transmitted from sexual interactions (Knell and Webberley 2004).
171 Convergent changes were also linked to sex determination (primary sex determination; soma,
172 primary response to X:A ratio), which may control changes in the expression of sexual traits, and
173 several metabolic processes. In whole-body samples we find some reproduction associated terms
174 (courtship behavior, male mating behavior, male courtship behavior, sperm storage, regulation of
175 ovulation) as in the reproductive tract, and behavioral, and immune related terms (immune
176 response-regulating cell surface receptor signaling pathway) as in legs, but also some unique
177 terms relating to the cuticle (ecdysone, pupal chitin-based cuticle development).
178

179 ***Convergently expressed genes in whole-bodies show evidence for sexual trait decay***
180
181 Several of the enriched functional processes described above are suggestive of sexual trait
182 decay. Under this scenario we expect a reduction of purifying selection on genes underlying
183 sexually dimorphic traits in asexual species, indicated by an increased accumulation of non-
184 synonymous changes.
185
186 The power to detect differences in pN/pS or dN/dS between gene sets in asexuals is low, as
187 genes are inherited as a single linkage group. Nevertheless, we found that genes showing
188 convergent changes in expression in whole-bodies showed elevated pN/pS and dN/dS when
189 compared to the genomic background (permuted t-test p-value for: pN/pS < 0.0001, dN/dS =
190 0.0084, Fig. 3, Supplemental Figs. 3 and 4), consistent with the idea of sexual trait decay. Sexual
191 trait decay is further supported by the examination of functional annotations for such genes which
192 include one gene (OG-2854) that is produced primarily in male accessory glands in *Drosophila*,
193 and at least three other genes (OG-2197, OG-663, OG-1014) that are involved in pigment
194 synthesis pathways (pigmentation is sexually dimorphic in *Timema* see Fig. 1B). In contrast,
195 genes showing convergent changes in expression in the reproductive tract and legs did not show
196 elevated pN/pS or dN/dS (Fig. 3, Supplemental Figs. 3 and 4), suggesting that convergent
197 expression changes in these tissues do not coincide with reduced purifying selection acting on
198 their sequences.
199
200 We conducted several additional analyses to check the robustness of our results and corroborate
201 our interpretations. Firstly, we examined in detail the associated functional annotations of
202 candidate gene sets for which there was very strong evidence for convergent changes, and
203 secondly, we used cross-species mapping to examine expression changes occurring across the
204 whole transcriptome, rather than only in the subset of genes we identified as single copy orthologs
205 between the 10 species. Both approaches support the results from our original analyses and are
206 described below.
207
208 ***Strongly convergent candidate genes and their function***
209
210 Although all the convergent genes we identified showed an overall shift in expression across the
211 five species-pairs, often expression change in one or two of the pairs was small (<1.2 fold
212 change). We defined top candidate genes as convergent genes for which the absolute $\log_2$ fold
213 change in expression was more than 0.25 (~1.2 fold change) for all species-pairs. Most of these
214 top genes showed convergent shifts in the reproductive tract (36 genes, relative to 4 and 15 genes
215 for whole-body and legs, respectively) (Figure 4, Supplemental Table 1). The functions of these
216 candidate genes largely reflected the functional processes identified for the full set of convergently
217 expressed genes, and highlight a number of key genes potentially involved in producing asexual
218 phenotypes.
219
220 For the reproductive tract four genes are involved in meiotic spindle formation and centrosome
221 organization (OG-513, OG-1448, OG-1488, OG-314). In particular we find two genes (OG-1448,
222 OG-1488) belonging to a family of Elovl proteins that mediate elongation of very-long-chain fatty

223 acids, including an ortholog to *D. melanogaster* gene *bond*, which effects spindle formation and
224 has been shown to be important for meiotic, but not mitotic, cytokinesis (Szafer-Glusman et al.
225 2008). In particular, *D. melanogaster* males defective for *bond* commonly display two to four nuclei
226 in spermatids causing sterility. Female *bond* mutants are also infertile (Szafer-Glusman et al.
227 2008), although the mechanism is unknown. The other two genes (OG-513, OG-314) have roles
228 in centrosome function, including an ortholog to *poc1* which is involved in centrosome formation
229 (Blachon et al. 2009). Six genes (OG-758, OG-2002, OG-1478, OG-1993, OG-2686, OG-148)
230 were annotated with reproduction associated terms which may be responsible for the convergent
231 reproductive changes we observe between asexual and sexual females. Interestingly, one gene,
232 OG-511, is an ortholog to glucose dehydrogenase which is important for sperm storage in female
233 *D. melanogaster (Bloch Qazi et al. 2003)*. Finally, we find that 11 genes (OG-1195, OG-1478,
234 OG-1841, OG-2197, OG-2808, OG-366, OG-445, OG-511, OG-705, OG-712, OG-758, OG-810)
235 have annotations to the nervous system. The majority of these appear to be sensory in nature,
236 and in particular seven are annotated with the GO term "sensory perception of pain". Changes in
237 these genes may represent changes associated with female receptivity and post-mating
238 behaviour in asexual females, which are target of substances in the male ejaculate (Heifetz and
239 Wolfner 2004; Sakai et al. 2009; Heifetz et al. 2014).
240
241 For leg samples three genes (OG-1651, OG-2048 and OG-1081) are involved in immune defence.
242 In particular orthologs of both genes (*Trx-2* and *MP1*) are involved in the activation of melanisation
243 in response to fungal and bacterial infection in *D. melanogaster* (Tang et al. 2006; Jin et al. 2008).
244 Three genes are involved in cuticle development (OG-2221, OG-2738, and OG-2995). Orthologs
245 of two other genes (OG-1371 and OG-2031) are involved in male specific behaviours (male
246 courtship behavior and inter-male aggressive behavior) in *D. melanogaster* (*CaMKII* and *Fkbp14*)
247 (Mehren and Griffith 2006; Edwards et al. 2009). Since these genes are also expressed in
248 females, changes to their expression may have resulted from the release of intralocus sexual
249 conflict.
250
251 Whole-body samples had only four strong candidate genes, and all either have no annotation or
252 have only broad GO-terms annotated. One potentially interesting gene, OG-2188, has an ortholog
253 (*CG12237*) that has been associated with female sterility in *D. melanogaster* (Sopko et al. 2014).
254 Finally, the remaining candidate genes across all tissues were either unannotated (12 genes) or
255 only have very broad GO-terms annotated (10 genes).
256
257 ***Cross-species mapping***
258
259 Using only the 3010 genes with 1-to-1 orthologs across all species could impact our ability to
260 detect convergent changes since we only use a relatively small fraction of the total number of
261 transcripts in each assembly (23435 to 37847; Supplemental Table 9). To investigate more genes,
262 we mapped reads from all samples to genes from each species which had a reciprocal-best-blast-
263 hit between sexual-asexual sister species (which includes the 1-to-1 orthologs analysed above).
264 This approach generated 10 different datasets (one for each species assembly), with between
265 15500 and 17583 genes. After filtering out genes with low expression (using cpm, see Methods)
266 in each dataset, this approach allowed us to examine between 2.43-3.12 (dependent on species

267 and tissue) times more genes than using the 1-to-1 orthologs (Supplemental Table 9). Results
268 from this approach qualitatively confirmed the results found using only the 1-to-1 orthologs: the
269 percentage of genes showing a convergent expression ranged from 4-5% for whole-body samples
270 and 6-8% for the reproductive tract and leg samples, dependent on which of the species
271 transcriptome was used (Supplemental Table 9), and GSEA produced similar enriched GO terms
272 (Supplemental Tables 10-12)
273
274 ***Species-pair specific changes***
275
276 The approach taken above allowed us to identify genes which showed convergent changes in
277 expression across independent transitions to asexuality. This approach will not identify expression
278 changes confined to a single or a few species-pairs. Changes occurring in only a minority of
279 species-pairs are clearly not convergent at the gene expression level, however, these changes
280 could be convergent at the functional process level, whereby species-pair specific changes in
281 gene expression are involved in common functional processes between species-pairs (Rittschof
282 et al. 2014; Berens et al. 2015). To test this, we compared each asexual species to its closest
283 sexual relative and called differentially expressed (DE) genes from each pairwise comparison.
284
285 The number of significantly DE genes between each pair varied greatly depending on species-
286 pair and tissue, with a generally greater number of genes DE in leg tissue (59 to 626,
287 Supplementary Fig. 5). This greater number in leg tissue is likely due to the smaller variation
288 between replicates (common biological coefficient of variation was lowest for legs: whole-body =
289 0.314, reproductive tract = 0.340, legs = 0.238), as tissue differences disappeared when a fold-
290 change threshold was applied (Supplementary Fig. 6). There were no genes that showed overlap
291 between all sexual-asexual species-pairs in any tissue (Fig. 5A). Examination of overlaps
292 between pairs of sexual-asexual species-pairs found some overlapping genes, but these were
293 close to the expectation by chance (Fig. 5B, for all levels see Supplemental Table 13). The
294 majority of the DE genes also showed a significant interaction between species-pair and
295 reproductive mode in the model used to identify convergently changing genes (whole-body = 69%,
296 reproductive tract 66%, and legs = 81%), corroborating the finding that the vast majority of the DE
297 genes are species-pair specific. Note the species-pair by reproductive mode interactions do not
298 appear to be generated by one specific species-pair as generally genes DE between one species-
299 pair were not DE between the other 4 species-pairs.
300
301 The DE genes of the different species pairs are not involved in convergent functional processes.
302 Species-pair-specific genes were enriched for a number of GO terms (presented in Supplemental
303 Table 14), however no GO terms were found to overlap between all pairs in any tissue
304 (Supplemental Fig. 7). Examination of overlaps between pairs of sexual-asexual species-pairs
305 found some overlapping GO terms, but these were close to the expectation by chance
306 (Supplemental Table 15). This pattern remained even when a more liberal approach, whereby
307 related GO-terms were considered as a unit, was applied (Supplemental Fig. 8A). This overall
308 lack of overlap suggests that species-pair-specific genes are not involved in producing convergent
309 phenotypes. Instead, species-pair-specific genes are the product either of lineage-specific
310 selection (for instance, if different species use different mechanisms to achieve parthenogenesis)

311 or of drift. These two processes are difficult to disentangle, but our results are more consistent
312 with drift rather than with lineage-specific selection. Indeed, species-pair specific genes showed
313 similar changes in gene expression across tissues, in contrast to the mainly tissue-specific
314 changes uncovered for convergently changing genes. The overlap of species-pair specific genes
315 between tissues was significantly greater than expected by chance (Table 1).
316
317 Finally, these results were reproduced when examining a much larger set of genes (genes with
318 reciprocal-best-blast-hits between species-pairs, see above) as both genes DE between each
319 species-pair, and their enriched GO terms, showed little overlap (Supplemental Figs 9 and 10,
320 and Supplemental Tables 16 and 17).

Discussion

Asexuality has convergently evolved numerous times across the tree of life, and a large body of research focuses on the reasons why sexual reproduction persists in the face of competition from asexual lineages. By contrast, the molecular underpinnings of transitions from sexual to asexual reproduction remain largely unknown (Neiman et al. 2014). In this study we examined gene expression changes associated with transitions to asexuality across five independently evolved asexual lineages, in whole-bodies, reproductive tracts and legs. The changes we observe provide, for the first time, insights into the convergent evolution of asexuality at the molecular level.

We found evidence for convergent changes in gene expression in all three tissues. Four lines of evidence suggest that these changes are a product of selection. Firstly, parallel changes across multiple independent transitions represent strong evidence of selection and thus are unlikely to be due to drift (Losos 2011; Natarajan et al. 2016). Secondly, convergent changes were primarily tissue-specific. This finding is consistent with selection, because expression changes due to drift are likely to be correlated across tissues (Blekhman et al. 2008; Liang et al. 2018). Indeed, the different functional roles of reproductive tracts and legs make it unlikely that selection would drive changes in the same genes in all tissues. Thirdly, expression changes of convergent genes inferred over the *Timema* phylogeny fit better with a model of adaptive change than with a model of simple drift. Finally, the functional processes of convergently expressed genes mirror the changes observed at the phenotypic level, supporting the interpretation that these genes contribute to the convergent phenotypic changes observed between sexual and asexual females.

The overall amount of convergence is striking, particularly in the reproductive tract and legs with approximately 8% of genes showing a convergent shift in expression. Such a large amount of convergence suggests that the path from sexual reproduction to asexuality is strongly constrained, requiring changes to the same genes and biological processes in order to produce asexual phenotypes.

Convergent changes in gene expression reveal the mechanisms underlying the production of asexual offspring

Asexuality is a complex adaptation that includes two major components: the ability to produce viable asexual offspring, and secondary adaptive changes that would not have been selected for in sexual species (e.g. the reduction of costly sexual traits). A key change necessary for the production of asexual offspring is the ability to produce unreduced eggs (Engelstädter 2008). Convergently expressed genes in the reproductive tract were enriched for changes in meiosis, and in particular meiotic spindles, which are key for the proper division of cells during meiosis. Mutations in meiotic spindles have been shown to result in unreduced meiotic products in *D. melanogaster*, and specifically in two genes (*bond* (Szafer-Glusman et al. 2008) and *pelo* (Eberhart and Wasserman 1995)) which show convergent changes in expression in asexual *Timema.* As such we suggest that these changes may underlie the non-reduction of eggs in asexual *Timema.* An alternative hypothesis is that since *Timema* reproduce parthenogenetically (and thus likely no longer recombine) changes in meiotic genes represent trait decay. Although

365 possible, previous work has shown that, in fact, meiotic genes are not only retained in asexual
366 lineages without damaging mutations, but often appear to be subject to selection for changes in
367 expression, via duplication or differential upregulation of promoters (Srinivasan et al. 2010;
368 Hanson et al. 2013; Raborn et al. 2016; Warren et al. 2018). Taken together with our results, we
369 suggest that modifications to meiotic genes, specifically those that that disrupt meiotic cell
370 division, are key in overcoming a major barrier to the evolution of asexuality: the production of
371 unreduced eggs.
372
373 The production of unreduced eggs is not the only barrier to producing offspring asexually. In most
374 species, sperm transfer essential components for the formation of a functioning centrosome
375 (Schatten 1994; Manandhar et al. 2005). This paternal contribution represents a second key
376 barrier in the evolution of parthenogenesis in many systems (Engelstädter 2008). However, in
377 phasmids the centrosome is assembled without any contribution from sperm in both sexual and
378 asexual species (Marescalchi et al. 2002). This may act as pre-adaptation for asexuality in stick
379 insects and account, in part, for the large number of asexual stick insects.
380
381 A final barrier to asexual offspring production in many systems is egg activation. In many species
382 mature oocytes are arrested at a specific stage (e.g. at metaphase II in mammals, and metaphase
383 I in most insects), and must be activated by sperm to re-enter the cell-cycle (Stricker 1999;
384 Engelstädter 2008; Nishiyama et al. 2010). In insects however, egg activation does not require
385 sperm as activation is induced by the transit through the reproductive tract (Sartain and Wolfner
386 2013). Despite this, ovulation and egg-laying rates are strongly tied to mating (Eberhard 1996;
387 Gillott 2003) meaning this signal must be modified in order for asexual insects to have normal
388 levels of fecundity. In insects, the signal to a female that she has successfully mated is likely
389 detected by sensory neurons in her reproductive tract (Yapici et al. 2008). Consistent with this,
390 we find changes in gene expression linked to sensory neurons in the reproductive tract of asexual
391 females, which may act to cue high levels of ovulation without mating. Alternatively, these
392 changes may represent the decay of these neurons since they are no longer needed to detect
393 mating events, or these changes may result from cessation of sexual conflict. Sensory neurons
394 in the reproductive tract are known targets of substances in the male ejaculate (Heifetz and
395 Wolfner 2004; Sakai et al. 2009; Heifetz et al. 2014) to induce the release of eggs and to reduce
396 female receptivity (Gillott 2003). This manipulation is countered by female resistance adaptations
397 which are likely costly, meaning that, following a transition to asexuality, there will be selection
398 against them.
399
400 ***Convergent changes in gene expression show evidence for the decay of female sexual***
401 ***traits***
402
403 Sexual traits in asexual females are often observed to be reduced or lost (van der Kooi and
404 Schwander 2014). For instance, in insects, females typically produce pheromones as a sexual
405 cue to attract males (Greenfield 2002), and this cue has been repeatedly reduced or lost in several
406 asexual species (see van der Kooi and Schwander (2014)), including *Timema* (Schwander,
407 Crespi, et al. 2013). Such trait decay can be the result of either reduced purifying selection acting
408 on traits that are now selectively neutral, or selection to reduce the cost of producing sexual traits.

409 In asexual *Timema* reproductive decay has been primarily attributed to selection rather than
410 reduced purifying selection, as reproductive trait decay in very young asexual lineages is as
411 extensive as in old ones (Schwander, Crespi, et al. 2013).
412
413 Convergent gene expression changes underlying the decay of reproductive traits are mostly
414 observed in *Timema* whole-bodies. In particular, we find enrichment of terms associated with
415 sperm storage and sexual behaviour. Changes in the legs were less obviously associated with
416 reproductive trait decay, however we do find changes in genes involved in cuticle development,
417 pigment biosynthesis, sensory perception of touch, and changes in sexual behavior. These
418 changes could represent the reproductive decay of both sexual cues (e.g. cuticular hydrocarbons
419 and pigmentation which are both important for mate choice in insects (reviewed in Hunt and
420 Sakaluk (2014)), and their detection (via sensory receptors on the leg (reviewed in Dahanukar et
421 al. (2005)). In addition, we also find changes in genes associated with sex determination in the
422 soma, including *sex-lethal*, a master-feminizing switch in *Drosophila* (Cline et al. 2010) which may
423 have a major influence the development of many sexual traits in the legs.
424
425 Although we focus on expression, it is possible that the decay of sexual traits is also evident at
426 the sequence level. By examining the coding regions of genes, we found evidence for reduced
427 purifying selection acting on the sequence of genes showing convergent expression changes in
428 the whole-body. This suggests, that in some cases, the reduction of sexual traits may be
429 accomplished by both expression and sequence changes, which potentially act interactively to
430 produce a phenotypic change.
431
432 Unexpectedly, we also find changes to immune function in the legs and whole-body, the majority
433 of which show down-regulation in asexual females. A possible explanation for this is that asexual
434 females are likely to face a reduced number of immune challenges compared to sexual females
435 due to the elimination of sexually transmitted diseases, the costs of which can be considerable,
436 even shaping the evolution of many aspects of an organism's life history, such as mate choice,
437 mating rate, and sexual signal investment (Kokko et al. 2002; Knell and Webberley 2004). As
438 such we suggest asexual females may be reducing the allocation of resources to immune function
439 due to the absence sexually transmitted diseases. This effect may be particularly strong in solitary
440 species such as *Timema*, where the majority of socially transmitted diseases come from sexual
441 interactions.
442
443 ***Species-pair specific changes***
444
445 In addition to convergent changes, we also identified many species-pair specific gene expression
446 changes. In contrast to convergent genes, species-pair specific genes showed common shifts in
447 expression across tissues, and inconsistent associations with functional processes between
448 species-pairs, that were largely unrelated to asexual phenotypes. Taken together, these results
449 suggest that the majority of changes we observe from a single sex-asex species-pair comparison
450 are due to drift rather than selection. Our findings thus highlight the problem of drawing inferences
451 on the causes or consequences of asexuality from the examination of only a single transition to

452 asexuality, whereas examining several transitions allows us to disentangle adaptive changes and
453 those due to drift.
454
455 Overall, we find evidence for a striking number of convergent changes across five transitions to
456 asexuality. Previous studies that have examined convergent expression changes across the
457 genome have found little evidence of convergence at the gene level (<1%) (Rittschof et al. 2014;
458 Berens et al. 2015), underlining the surprisingly large amount of convergent gene expression (up
459 to 8%) we find here. The amount of molecular convergence to expect, however, is dependent on
460 several factors including the complexity of the phenotype, and the size of the mutational target
461 (Martin and Orgogozo 2013). For example, we find that a key change required for asexual
462 reproduction, the production of unreduced eggs, likely requires changes to meiotic spindle
463 regulation. The pathways that govern meiotic spindle regulation are relatively small in number
464 (Bennabi et al. 2016), meaning that only a small minority of genes are likely able to confer the
465 relevant changes, making the chance of molecular convergence for this trait relatively high. By
466 contrast, the observed reduction of sexual traits could be produced by changes to numerous
467 genes and pathways (i.e. there is a large mutational target) making convergent molecular
468 changes for these traits less likely. Despite this, our and previous studies examining trait loss
469 have also demonstrated a high amount of convergence (Whittall et al. 2006; Gross et al. 2009;
470 Partha et al. 2017; Sackton et al. 2018), implying that certain genes have a disproportionate role
471 in not only the convergent evolution of novel phenotypes, but also in their convergent loss (Martin
472 and Orgogozo 2013; Stern 2013).

Methods

Samples

Females for whole-body samples were collected from the field as juveniles in spring 2013. All individuals were then raised in common garden conditions (23°C, 12h:12h, 60% humidity, fed with *Ceanothus* cuttings) until eight days following their final molt. Prior to RNA extraction, individuals were fed with artificial medium for two days to avoid RNA contamination with gut content and then frozen at -80°C. Individuals used for tissue-specific samples were collected in spring 2014 as juveniles and raised in the same common-garden conditions as whole-body samples. For leg samples three legs were used from each individual (one foreleg, one midleg, and one hindleg). Reproductive tracts were dissected to consist of ovaries, oviducts and spermatheca. Note the same individuals were used for leg and reproductive tract samples. Collection locations for all samples are given in Supplemental Table 18.

RNA extraction and sequencing

The three biological replicates per species and tissue consisted of 1-9 individuals per replicate, which were combined prior to RNA extraction (207 individuals in 90 replicates in total; see Supplemental Table 18). RNA extraction was performed by freezing samples in liquid nitrogen followed by addition of Trizol (Life Technologies) before being homogenized using mechanical beads (Sigmund Lindner). Chloroform and ethanol were then added to the samples and the aqueous layer transferred to RNeasy MinElute Columns (Qiagen). RNA extraction was then completed using an RNeasy Mini Kit following the manufacturer's instructions. RNA quantity and quality was measured using NanoDrop (Thermo Scientific) and Bioanalyzer (Agilent). Strand-specific library preparation (1 library per replicate) and single-end sequencing (100 bp, HiSeq2000) were performed at the Lausanne Genomic Technologies Facility.

The 90 libraries produced a total of just over 3 billion single-end reads. Four whole-body and six tissue-specific libraries produced significantly more reads than the average for the other samples. To reduce any influence of this on downstream analyses, these libraries were sampled down to approximately the average number of reads for whole-body or tissue-specific libraries respectively using seqtk (https://github.com/lh3/seqtk Version: 1.2-r94).

Transcriptome references

De novo reference transcriptome assemblies for each species were generated previously (Bast et al. 2018). For our analyses we used the 3010 one-to-one orthologs present in all 10 *Timema* species as identified by Bast et al. (2018). Identified ortholog sequences varied in length among different species. Since length variation might influence estimates of gene expression, we aligned orthologous sequences using PRANK (v.100802, default options) (Löytynoja and Goldman 2005) and trimmed them using alignment_trimmer.py (Parker 2016) to remove overhanging gaps at the ends of the alignments. If the alignment contained a gap of greater than 3 bases then sequence preceding or following the alignment gap (whichever was shortest) was discarded. Three genes

were discarded at this stage as the trimmed length of sequence was <300 bp. These trimmed sequences were then used as reference transcriptomes for read mapping. Note that genes with significant Blast hits to rRNA sequences were removed from the transcriptome references prior to mapping.

Read trimming and mapping

Raw reads were trimmed before mapping. Firstly CutAdapt (Martin 2011) was used to trim adapter sequences from the reads. Reads were then quality trimmed using Trimmomatic v 0.36 (Bolger et al. 2014): first clipping leading or trailing bases with a phred score of <10 from the read, before using a sliding window from the 5' end to clip the read if 4 consecutive bases had an average phred score of <20. Following quality trimming any reads <80 bp in length were discarded. Quality-trimmed reads from each library were then mapped separately to the reference transcriptome using Kallisto (v. 0.43.1) (Bray et al. 2016) with the following options -l 210 -s 25 --bias --rf-stranded for whole-body samples and -l 370 -s 25 --bias --rf-stranded for tissue specific samples (the -l option was different for whole-body and tissue specific samples as the fragment length for these libraries was different).

Differential expression analysis

Expression analyses were performed using the Bioconductor package EdgeR (v. 3.18.1) (Robinson et al. 2010) in R (v. 3.4.1) (R Core Team 2017). Analyses were done separately for each tissue. Genes with counts per million less than 0.5 in 2 or more libraries per species were excluded from expression analyses. Normalization factors for each library were computed using the TMM method in EdgeR. To estimate dispersion we then fit a generalized linear model (GLM) with negative binomial distribution with the terms species-pair, reproductive mode and their interaction. We used a GLM likelihood ratio test to determine significance of model terms for each gene by comparing appropriate model contrasts. P-values were corrected for multiple tests using Benjamini and Hochberg's algorithm (Benjamini and Hochberg 1995), with statistical significance set to 5%. Using this approach, we classified genes as convergently differentially expressed when there was a significant effect of reproductive mode (FDR < 0.05) but no interaction effect of species-pair by reproductive mode (FDR > 0.05). Differentially expressed genes within each species-pair were identified using pairwise contrasts between each sexual and asexual pair.

To determine if genes DE within each species-pair and tissue show greater than expected number of overlapping genes we used the SuperExactTest package (v. 0.99.4) (Wang et al. 2015) in R which calculates the probability of multi-set intersections. When examining multiple intersections p-values were multiple test corrected using Benjamini and Hochberg's algorithm implemented in R.

To test if the observed number of convergent genes was significantly greater than expected by chance we performed a permutation test where, for the read counts of each gene, we randomly switched the assignment of reproductive mode (sexual or asexual) within a species-pair. Note that all biological replicates from a particular group were always assigned to the same

561 reproductive mode (i.e. In the event of a switch, all sexual replicates were assigned as asexual,
562 and vice versa). This process was repeated to produce 10,000 permuted data sets, which were
563 then run through the gene expression pipeline described above to generate a distribution of the
564 number of convergent genes we expect to find by chance.
565
566 ***Ornstein-Uhlenbeck based models***
567
568 Ornstein-Uhlenbeck (OU) based models were fit using the R package ouch (v. 2.11-1) (King and
569 Butler 2009). We fit two models to each convergently expressed gene: a drift-model where
570 changes are modelled as Brownian motion, and an adaptive-optima model where asexual species
571 had a different adaptive-optima than sexual species. A log-likelihood ratio test was used to
572 determine if the adaptive-optima model was a significantly better fit to observed expression values
573 than the drift-model, and p-values were corrected for multiple tests using Benjamini and
574 Hochberg's algorithm (Benjamini and Hochberg 1995), with statistical significance set to 5%.
575 Mean observed expression values ($\log_2$ counts per million) for each species and gene were
576 calculated using EdgeR (see above). We used RAxML (v. 8.2.8, options: -p 12345 -m GTRCAT
577 -T 6) (Stamatakis 2014) to produce a maximum likelihood tree for use in ouch using the
578 concatenated one-to-one ortholog alignments produced in Bast et al. (2018).
579
580 ***GO term analysis***
581
582 Genes were functionally annotated using Blast2GO (version 4.1.9) (Götz et al. 2008) as follows:
583 sequences from each sexual species were compared with BlastX to either NCBI's nr-arthropod
584 or *Drosophila melanogaster* (drosoph) databases, keeping the top 20 hits with e-values $<1 \times 10^{-}$
585 ^{3}. Interproscan (default settings within Blast2GO) was then run for each sequence, and the results
586 merged with the blast results to obtain GO terms. This produced two sets of functional
587 annotations, one derived from all arthropods and one specifically from *Drosophila melanogaster*.
588 The *D. melanogaster* GO term annotation generated around four times more annotations per
589 sequence than NCBI's nr-arthropod database. We therefore conducted all subsequent analyses
590 using the GO terms derived from *D. melanogaster*, but note that results using the annotations
591 from all arthropods were qualitatively the same (see Supplementary Fig. 8B).
592
593 We conducted gene set enrichment analyses using the R package TopGO (v. 2.28.0) (Alexa and
594 Rahnenfuhrer 2016) using the elim algorithm to account for the GO topology. Gene set
595 enrichment analyses identify enriched GO terms in a threshold-free way, by finding GO-terms that
596 are overrepresented at the top of a ranked list of genes. For comparisons within a species-pair,
597 genes were ranked by FDR; to identify enrichment of convergent genes, genes were ranked by
598 FDR value for reproductive mode, with the FDR value for genes that showed a significant lineage
599 by reproductive mode set to 1. GO terms were considered to be significantly enriched when $p <$
600 0.05. Enriched GO terms were then semantically clustered using ReviGO (Supek et al. 2011) to
601 aid interpretation.
602
603 The significance of overlapping GO terms was determined using SuperExactTest as described
604 above. The hierarchical nature of GO terms generates a bias towards finding a significant amount

605 of overlap, since enrichment terms are non-independent. It is however possible that the
606 complexity of the GO term hierarchy could lead to convergent functional processes being
607 overlooked. For instance if a GO term is enriched in one comparison, but its parent term is
608 enriched in another comparison, then there would be no apparent overlap. To address this, we
609 also looked at the amount of 'linked overlap' of GO terms, whereby significant GO terms were first
610 clustered together based on parent or child terms.
611
612 For the GO term enrichment analyses of convergently differentially expressed genes we used
613 only the annotation from *T. bartmani* as it had the most number of sequences annotated.
614 Annotations to each of the other species were very similar to those from *T. bartmani,* with 80% of
615 annotations being identical across all 5 species annotations. The remaining 20% of sequences
616 were typically characterized by an additional term in one or more of the species. For comparisons
617 within a lineage we used the annotation of the sexual species in that lineage. Although the
618 annotations are very similar across all ten species the small differences in annotation could create
619 differences in the amount of overlap observed between contrasts (e.g. if a term is annotated to
620 an ortholog in one annotation but not another). To examine this, we repeated the analysis using
621 only annotations from *T. bartmani*. This produced a virtually identical result (Supplemental Fig.
622 8C) as when using the species-pair specific annotations.
623
624 ***Polymorphism and divergence***
625
626 To test for differences in the rate of evolutionary divergence between gene categories, we used
627 dN/dS ratios for each of the one-to-one orthologs from Bast et al. (2018). Briefly, Bast et al. (2018)
628 aligned each of the one-to-one orthologs with M-Coffee (Wallace et al. 2006) and trimmed them
629 with Gblocks (Talavera and Castresana 2007). These alignments were then used as input for
630 codeml of the PAML package (Yang 2007) to generate maximum likelihood estimates of dN/dS
631 for each terminal branch in the phylogeny (using the "free model"). To obtain an estimate for
632 pN/pS for each ortholog, reads from the whole-body libraries (based on a pool of three individuals)
633 for each asexual species were mapped to the reference using RSEM/bowtie2 with default
634 parameters and fragment length mean = 200 fragment length sd = 100 (Li and Dewey 2011;
635 Langmead and Salzberg 2012). Samtools v1.2 was then used to create an mpileup file, which
636 was filtered with VarScan v2.3.2 (minimum coverage = 20, minor allele frequency = 10%, and
637 minimum average phred quality = 20) to obtain SNPs. To identify nonsynonymous and
638 synonymous segregating polymorphisms we identified the n-fold degenerate positions following
639 Li et al. (1985) from which pN, pS and (pN/pS) could be calculated per gene. Comparison of mean
640 pN/pS and dN/dS between convergent and non-convergent (background) genes was conducted
641 using a permutation t-test (number of permutations = 10000) in R.
642
643 ***Cross-species mapping***
644
645 All of the above analyses used only the one-to-one orthologs. To examine a larger fraction of the
646 transcriptome we produced species-pair references by using a reciprocal blast between the
647 assemblies of sexual-asexual sister species (*T. bartmani* - *T. tahoe, T. cristinae* - *T. monikensis*,
648 *T. poppensis* - *T. douglasi*, *T. californicum* - *T. Shepardi*, and *T. podura* - *T. genevievae*) (blastN,

minimum e-val = 0.00001, minimum query coverage = 30%). Prior to this step potential contaminants were filtered from these by blasting transcripts to local versions of the nt (using blastN, default options except task = blastn, max_target_seqs = 10) and nr (using blastX, default options except, max_target_seqs = 10) databases (downloaded: 07/08/2016) using NCBI's blast client (v. 2.2.30+). Blast hits with an e-value > 0.0000001 were discarded. The remaining blast hits were used to assign a phylum to sequences if >=50% of Blast hits came from one phylum (in the event of a tie, the taxa with the highest e-value was used as a tiebreaker). Transcripts that were assigned to a non-arthropoda phylum were discarded (note that transcripts with no Blast hits or that blasted to mixed phyla were retained). This filtering removed between 4-8% of transcripts (see Supplemental Table 9). Reads of each species were then mapped to each species-pair references in the same way as for the 1-to-1 orthologs. Differential expression analyses and GO-term enrichment analyses were then repeated as described above.

Data

Raw reads have been deposited in the SRA. Accession codes are given in Supplementary Table 18. Scripts for the analyses in this paper are available at: https://github.com/DarrenJParker/Timema_convergent_gene_expression.

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Author contributions

T.S., D.J.P. and K.J. designed the study. Z.D., K.J. and TS collected samples, performed dissections and molecular work. D.J.P. analysed the data with input from J.B., T.S. and M.R.R. D.J.P. and T.S. wrote the manuscript with input from all authors.

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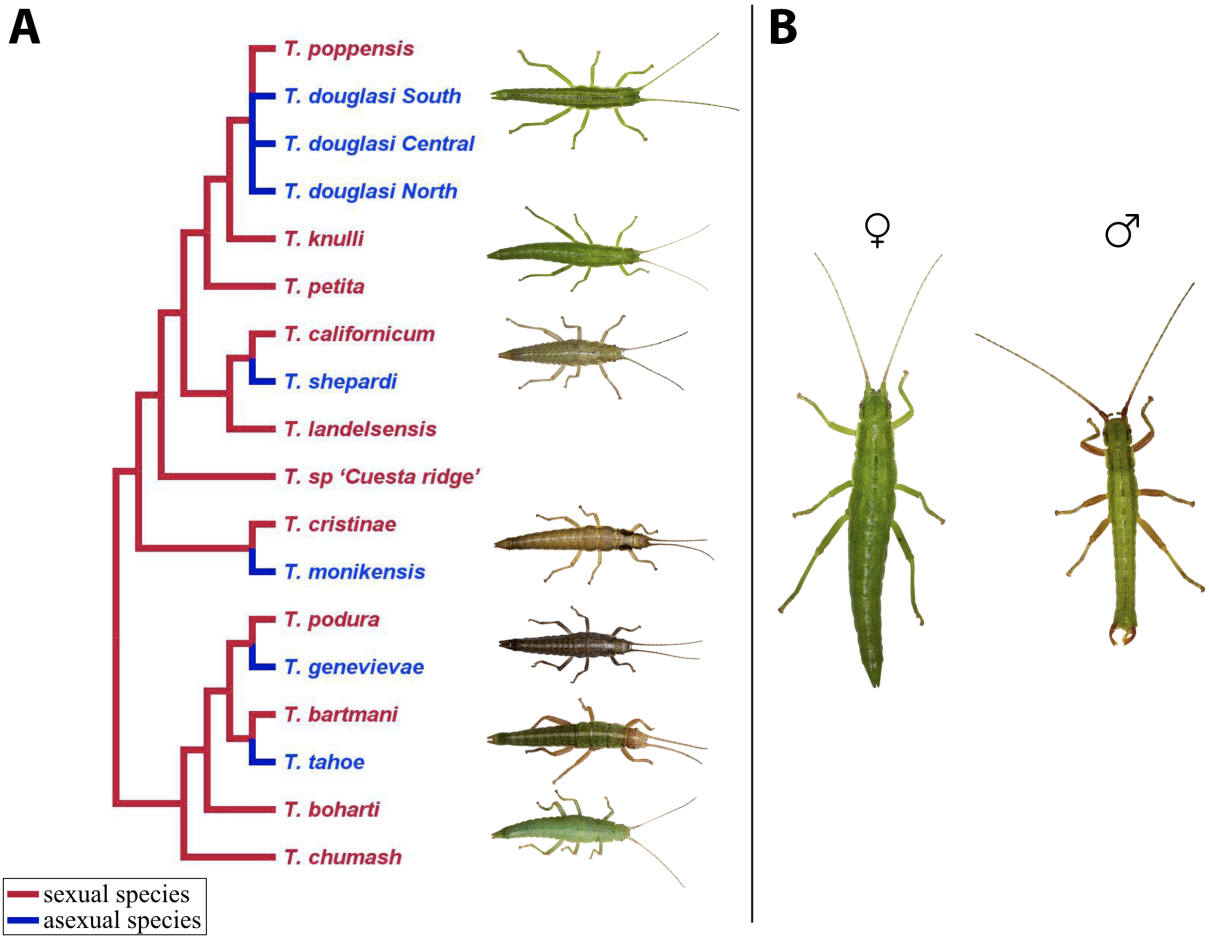
Table 1 | Overlap of differentially expressed genes between different tissue types. Number of genes expected by chance given in parentheses. P-values are from a fisher's exact test corrected for multiple tests. Species names are abbreviated as follows: Tbi = *T. bartmani*, Tce = *T. cristinae*, Tps = *T. poppensis*, Tcm = *T. californicum*, Tpa = *T. podura*, Tte = *T. tahoe*, Tms = *T. monikensis*, Tdi = *T. douglasi*, Tsi = *T. shepardi*, and Tge = *T. genevievae*.

	Reproductive tract & legs	**Whole-body & legs**	**Whole-body & reproductive tract**
Convergent genes	22 (16) p = 0.14	7 (5) p = 0.21	4 (4) p = 0.65
Species-pair (Tbi-Tte)	62 (24) p = 1.30 $\times 10^{-14}$	68 (29) p = 4.57 $\times 10^{-14}$	25 (7) p = 4.53 $\times 10^{-9}$
Species-pair (Tce-Tms)	88 (47) p = 1.36 $\times 10^{-10}$	48 (9) p = 6.19 $\times 10^{-25}$	47 (14) p = 3.86 $\times 10^{-16}$
Species-pair (Tcm-Tsi)	42 (11) p = 4.45 $\times 10^{-15}$	24 (5) p = 4.64 $\times 10^{-11}$	19 (3) p = 4.64 $\times 10^{-11}$
Species-pair (Tpa-Tge)	153 (134) p = 1.99 $\times 10^{-2}$	102 (60) p = 1.22 $\times 10^{-9}$	86 (54) p = 8.75 $\times 10^{-07}$
Species-pair (Tps-Tdi)	56 (20) p = 1.31 $\times 10^{-15}$	59 (37) p = 6.08 $\times 10^{-5}$	32 (7) p = 7.81 $\times 10^{-14}$

903 **Figures**



904
905 **Figure 1** | **A.** Phylogeny of described *Timema* species (redrawn from (2017) with asexual species
906 added from (2011)). Sexually reproducing species are shown in red, independently derived
907 asexual lineages in blue. Our study used the five asexual species (for *T. douglasi* only the
908 southern lineage was used (labelled as *T. douglasi* South)) and their sexual sister species. **B.**
909 Sexual dimorphism in *Timema* (*T. knulli*).
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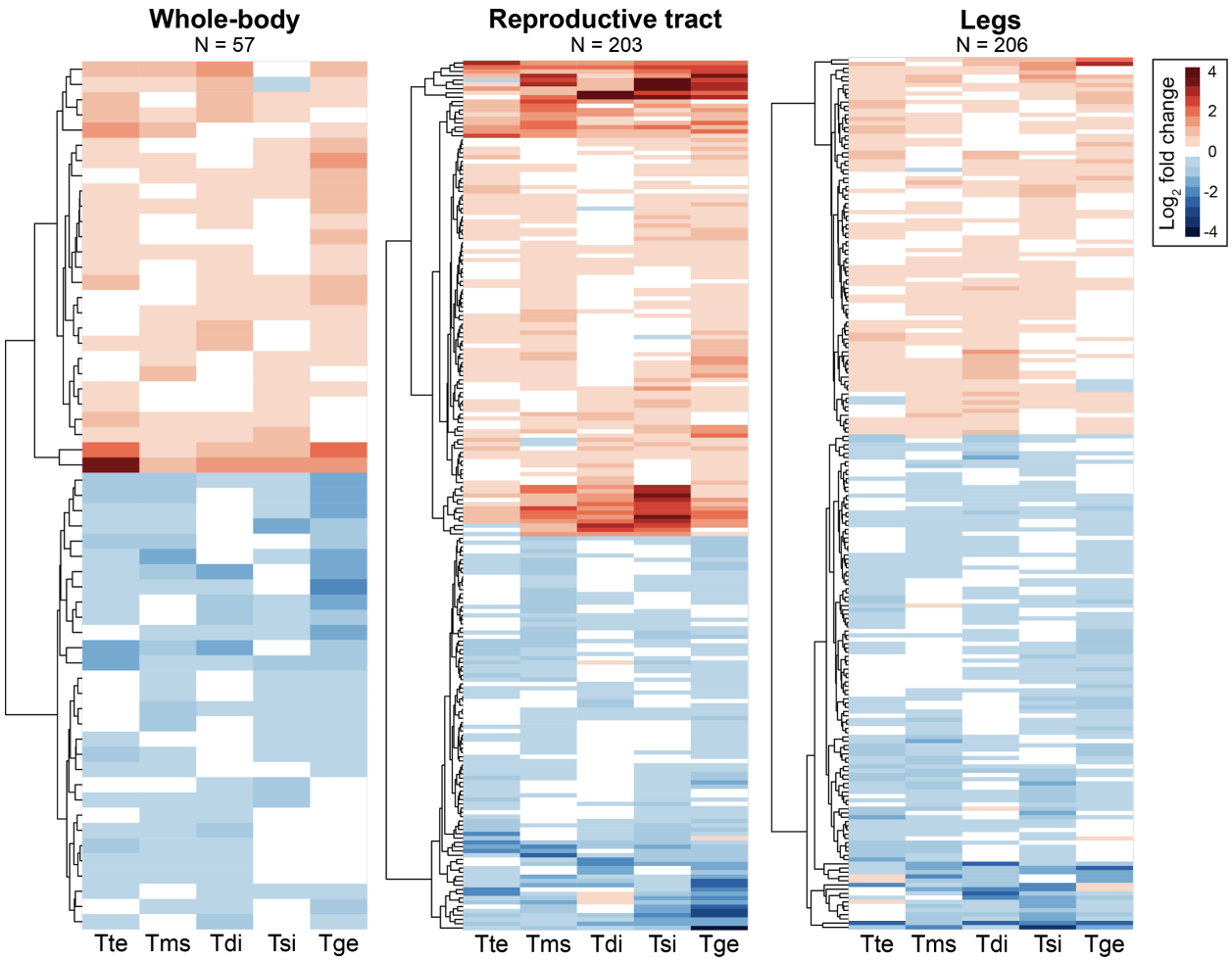

Figure 2 | Heatmaps of genes showing convergent gene expression changes between sexual and asexual females for whole-bodies, reproductive tract, and legs. Species names are abbreviated as follows: Tte = *T. tahoe*, Tms = *T. monikensis*, Tdi = *T. douglasi*, Tsi = *T. shepardi*, and Tge = *T. genevievae*.

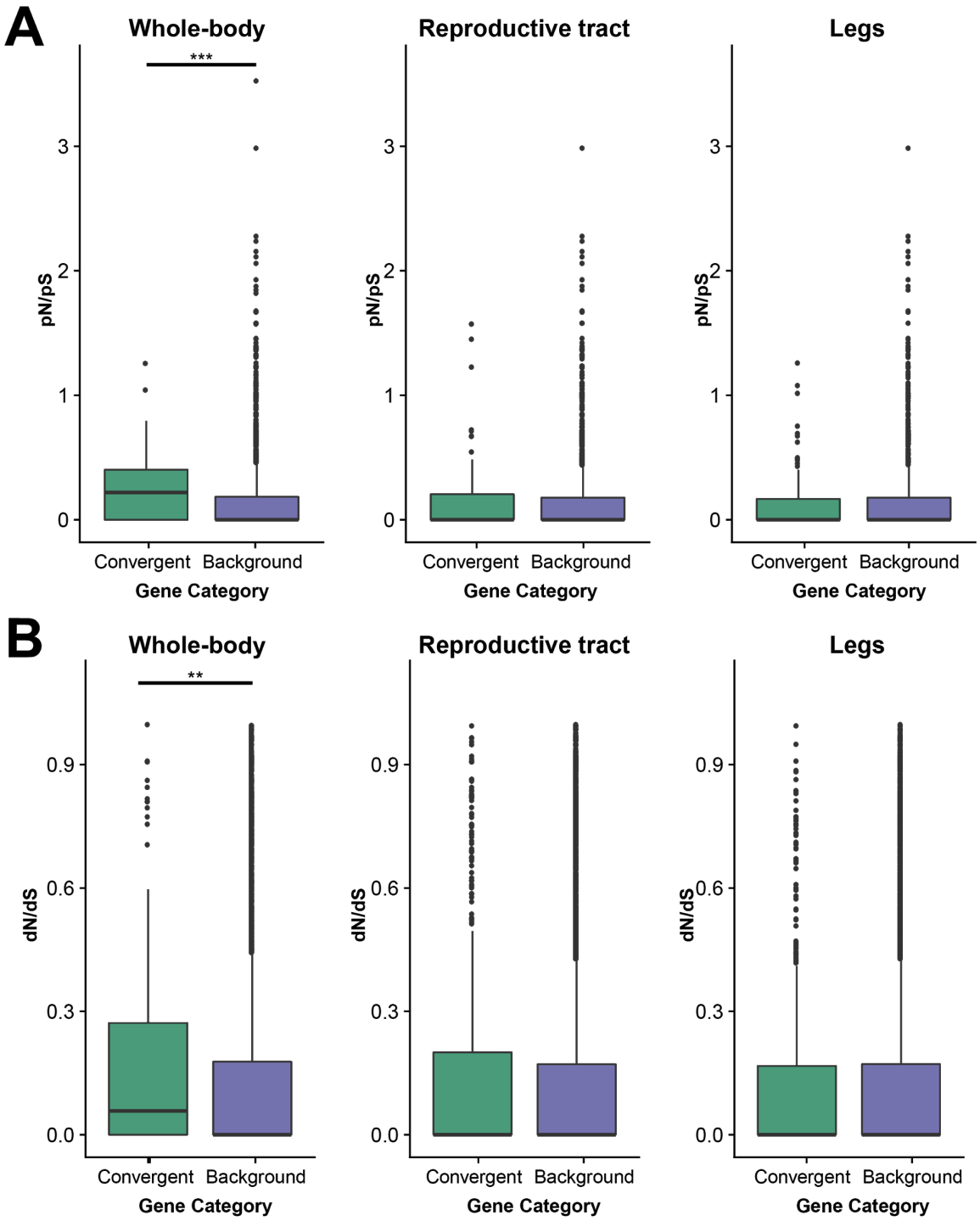

Figure 3 | pN/pS ratios (A) and dN/dS ratios (B) for convergently expressed genes versus all other genes expressed in that tissue for whole-bodies, reproductive tracts and legs. Significance is indicated by asterisks (** < 0.01, *** < 0.001).

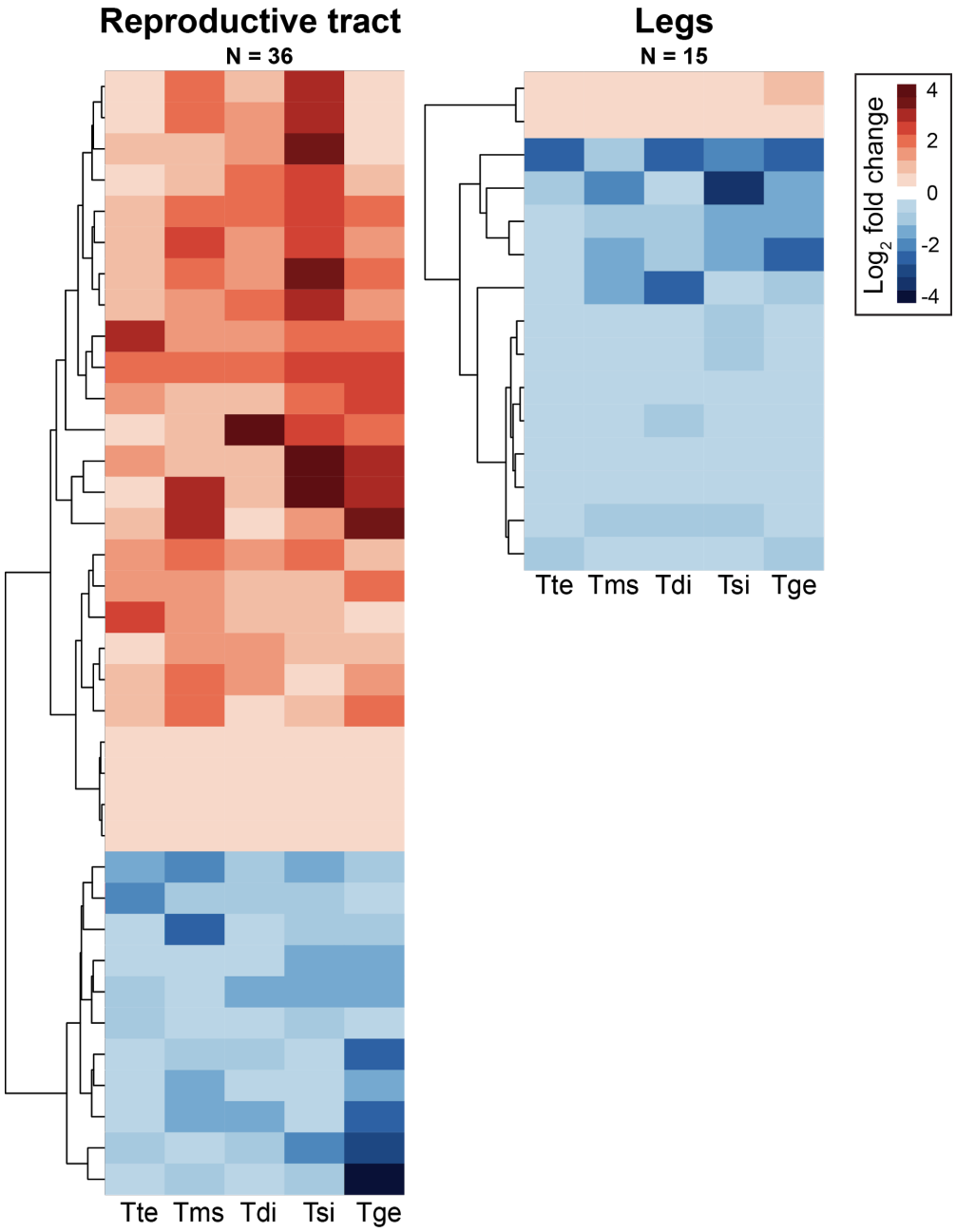

Figure 4 | Heatmaps of candidate convergent gene expression changes between sexual and asexual females for the reproductive tract and legs. Species names are abbreviated as follows: Tte = *T. tahoe*, Tms = *T. monikensis*, Tdi = *T. douglasi*, Tsi = *T. shepardi*, and Tge = *T. genevievae*.

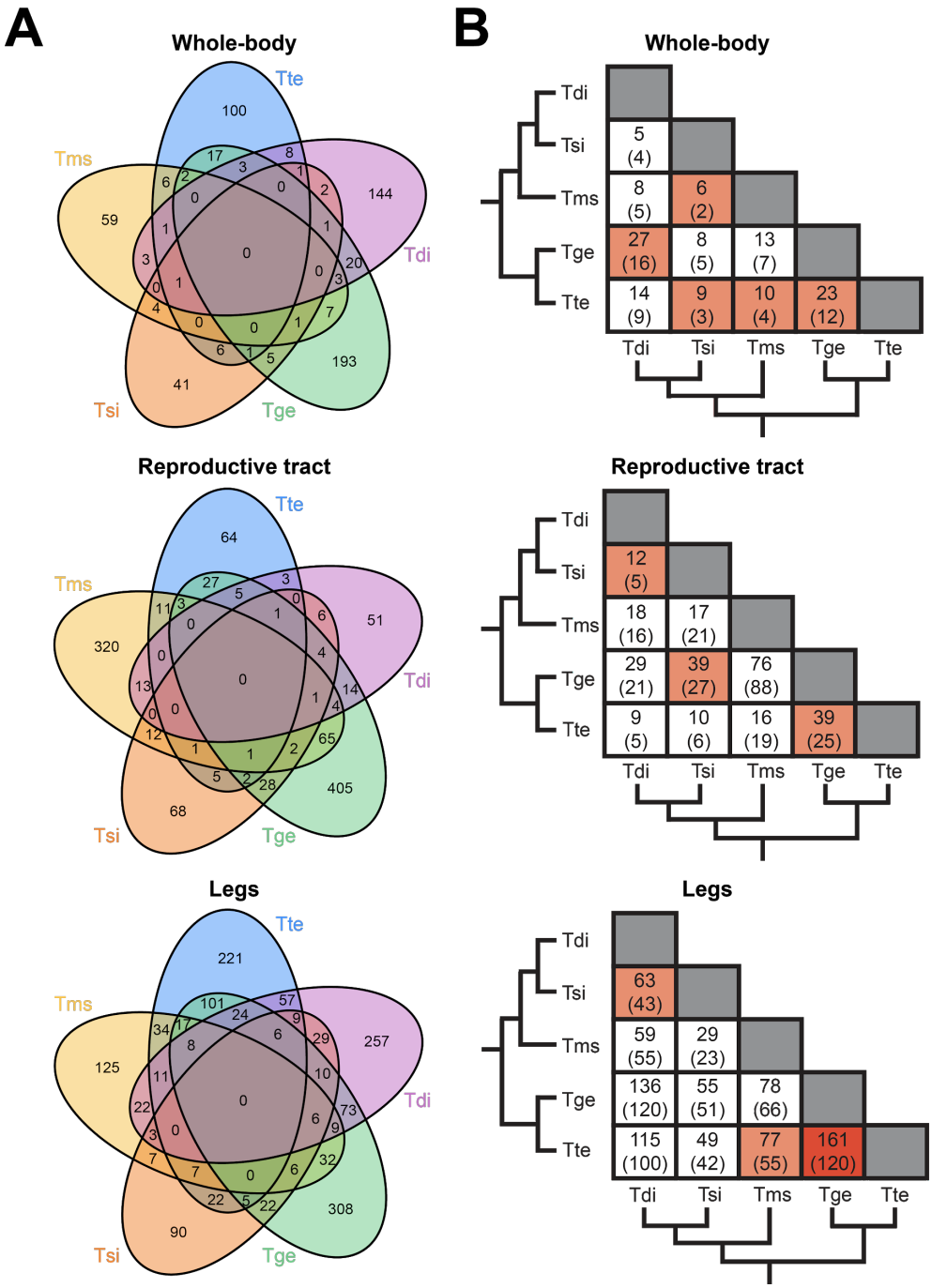

Figure 5 | A. Venn-diagrams showing the number differentially expressed (DE) genes between sexual and asexual females that are shared among species-pairs for whole-body, reproductive tract, and legs for 10 species orthologs (FDR < 0.05). B. Matrices showing pairwise overlap of DE genes between sex-asex species with the number of genes expected by chance given in parentheses. Colours represent a significantly greater overlap than expected by chance (red, FDR < 0.001, orange < 0.05). The phylogeny shows the relationships between asexual species (from (2011)). Species-pair names are abbreviated as follows: Tte = *T. tahoe*, Tms = *T. monikensis*, Tdi = *T. douglasi*, Tsi = *T. shepardi*, and Tge = *T. genevievae*.